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RESEARCH COMMUNICATION

Cytopathology, biology and molecular characterization of two Italian isolates of Malva vein clearing virus

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Abstract

Two Italian isolates of *Malva vein clearing virus* (MVCV), naturally infecting *Malva sylvestris* (common mallow) plants, were characterized at biological, cytopathological and molecular level. Experimental host range was comparable for both isolates and in agreement with those reported for other MVCV isolates. Cytopathology observed indicated type I of cylindrical inclusions caused by both isolates in common mallow. The 3' genome extremity of about 1800 nucleotides was sequenced for both isolates. It comprised of the 3' end of the NIb gene, the entire putative ORF of the coat protein (CP) and the 3' non-translated region of genome. Phylogenetic analysis based on CP gene did not shown any statistically significant grouping among ten different MVCV isolates, suggesting low level of variability among the MVCV isolates genetically characterized until now.

Keywords: *Malva sylvestris*; MVCV; inclusion bodies; molecular characterization; phylogeny

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Introduction

Malva vein clearing virus (MVCV) is a distinct member of the genus *Potyvirus* (family *Potyviridae*), according to particle morphology, cytopathology, vector transmission and genome analysis (Berger *et al.*, 2005; Andrew *et al.*, 2012). It was first described in *Malva sylvestris* (common mallow) in Germany (Hein, 1956). Natural spread of MVCV often occurs in species of *Malva* and *Lavatera* genera, but susceptible experimental host plants also include species belonging to *Abutilon*, *Althaea*, *Anoda*, *Gossypium*, *Hibiscus*, *Kitaibelia*, *Lavatera*, *Malva*, *Napaea*, *Sida*, *Sidalcea*, and *Urocarpidium* genera (ICTVdB Management, 2006). So far, no economic importance is attributed to MVCV, mainly because natural host range is limited to a few plant species belonging only to two genera within the Malvaceae family (ICTVdB Management, 2006). Up to date, no information are available on the biological and molecular variability of MVCV isolates in the world. The objective of this study was to characterize two field isolates of MVCV, causing vein clearing in common mallow in Southern Italy by host range, cytopathology and to assess phylogenetic relationships among different isolates of this virus based on the coat protein (CP) gene variability.

Materials and Methods

Virus isolates and host range. The two virus isolates characterized in this study were named DS-Ba-01 and Napoli. DS-Ba-01 isolate was obtained from a naturally infected common mallow plant collected in October 2001 in Southern Italy (Bari, Apulia region), showing severe symptoms of yellow mosaic and vein clearing (Fig. 1 A). Napoli isolate was obtained from naturally infected common mallow plant in March 2010 in Southern Italy (Napoli, Campania region), showing mosaic and vein clearing. Infected common mallow plants were evaluated by antigen-coated plate (ACP) ELISA using the potyvirus group-specific monoclonal antibody (Agdia, Inc., Elkhart,

IN), or by standard double-antibody-sandwich assay (DAS-ELISA) using antisera against *Cucumber mosaic virus* (CMV) and *Tomato spotted wilt virus* (TSWV), the latter previously reported to naturally infect *M. sylvestris* (Laviña *et al*., 2006).

Symptomatic leaves from both plants were used to prepare crude saps for mechanical inoculation, using described method (Parrella *et al*., 1998), of two sets of plants consisting into 10 plant species belonging to 5 botanical families: *Althaea rosea*, *Gossypium hirsutum, Malva sylvestris*, *M. neglecta*, *M. nicaensis*, *M. parviflora*, *Lavatera trimestris*, *Solanum lycopersicum*, *Nicotiana tabacum* cv. *Xanthi* nc, *Chenopodium quinoa*, *Cucumber sativus* cv. Marketer, *Phaseolus vulgaris* cv. Pinto. Inoculated plants were maintained in an insect-proof greenhouse and examined for symptom development up to three months post-inoculation (p.-i.). Virus identity was confirmed in symptomatic plants 40 days p.-i., by dot-blot hybridization assays using a MVCV specific riboprobe.

Electron microscopy. Crude extracts or partially purified virus preparations were observed with a Philips CM10 electron microscope (Eindhoven, The Netherlands) using pyroxilin-coated grids and 1% ammonium molybdate, pH 7.00, as a negative stain. Virus particles were measured using a carbon replica calibrated at 463 nm as a reference. For cytopathological studies, leaf pieces of 1 mm across were collected 3-4 weeks after inoculation on the intermediate fully expanded leaves (leaves above those inoculated and below the upper youngest leaves) from infected common mallow plants. Similar samples from healthy plants were used as control. Samples were fixed with gluteraldehyde, postfixed with osmium tetroxide, and embedded in araldite CY212 (Agar Scientific Ltd, Stansted, UK). Ultrathin sections were stained in 5% uranyl acetate and lead citrate, pH 12, before observations (Delécolle, 1978).

Nucleic acids purification, cDNA synthesis, cloning and sequence analysis. Total nucleic acids (TNAs) were extracted from 50 mg of DS-Ba-01-infected symptomatic young leaves (apical not fully expanded leaves) of common mallow, following the method described by White and Kaper (1989). TNAs were resuspended in 50 µl of sterile TE (Tris-EDTA, pH 7.4) and heated 5 min at 85°C prior to reverse transcription (RT). RT and polymerase chain reaction (PCR) were performed following a procedure derived from Gibbs and Mackenzie (1997), adapted by authors previously (Parrella *et al*., 2006). Amplicons were directly ligated into pGEMT Easy vector (Promega Corp., Madison, WI) and recombinant plasmids were used to transform competent cells of *Escherichia coli* DH5α strain. Plasmid DNA was purified from *E. coli* with Jet Quick Plasmid Miniprep Kit (Genomed, Löhne, Germany) and sequenced at MWG Biotech (Martinsried, Germany). Three clones were sequenced on both senses.

Phylogenetic analyses were conducted with the Italian and other isolates of MVCV, for which the nucleotide sequence of the CP genes were available in databank. The multiple sequence alignments were performed using the ClustalW program (Thompson *et al*., 1994). Phylogenetic analysis was carried out using the Maximum Likelihood algorithm implemented with MEGA software (Tamura *et al*., 2011). The best fit model of nucleotide substitution (Tamura-Nei) was determined by MEGA6 and 1000 bootstrap replicates were used.

Riboprobe production and dot blot assay. The two oligonucleotides MLSf (5'-GATCAACAAGCTCAGGAAGAG-3', forward primer) and MLSr (5'-CCTCACCATCAATCATAGTC-3', reverse primer) were designed based on the sequence obtained from the amplicon amplified with potyvirid 1 and potyvirid 2 primer pair. These primers were expected to amplify a fragment of 442 bp from the 5' of the putative CP of DS-Ba-02. PCR conditions included: initial denaturation at 94°C for 3 m, following by 35 cycles of 94°C for 45 s, 52 °C for 1 m and 72°C for 2 m. A final elongation step was of 72°C for 5 m. The amplicon was purified from agarose gel, cloned in pGEMT Easy Vector and sequenced. The sequence obtained was aligned with that obtained from the degenerate potyvirid primers to confirm the identity between the two sequences. The pMVCV recombinant plasmid containing the 442 bp fragment was then used to produce minus strand riboprobe against MVCV RNA following procedure described (Parrella *et al*., 2004).

Results and Discussion

Potyvirus-like particles were observed in leaf dips from common mallow plants collected in two Italian regions, and infected by DS-Ba-01 and Napoli isolates (Fig. 2 A and B). Their mean length was estimated to be 853 nm and 857 for DS-Ba-01 (n=27) and Napoli (n=30) respectively. Numerous cylindrical cytoplasmic inclusions were observed in cells of the epidermal, parenchymatic, or vascular tissues from infected plants. Depending upon the section orientation, inclusions appeared as pinwheels**,** scrolls and bundles (Fig. 1D and 1E). According to the types of cytoplasmic inclusions observed, consisting in pinwheels, scrolls (in cross section) and tubes (in longitudinal section), both DS-Ba-01 and Napoli isolates can be included in potyvirus subdivision I (Edwardson and Christie, 1996). Neither cylindrical cytoplasmic inclusions nor virus particles were observed in healthy plant samples. In serological tests, common mallow plants infected by DS-Ba-01 and Napoli isolates reacted positively only to the potyvirus group-specific monoclonal antibody, while no reaction was observed using CMV and TSWV specific antisera.

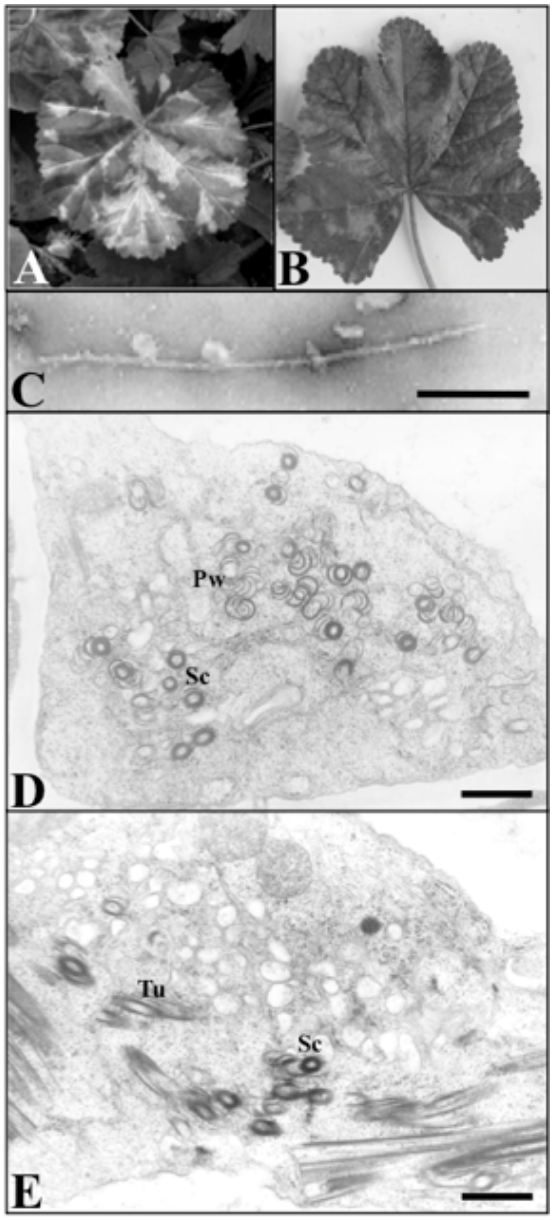

Fig. 1 - Symptoms elicited by DS-Ba-01 **(A)** and Napoli **(B)** MVCV isolates, naturally infecting *Malva sylvestris* plants. Negatively stained virus particle **(C)** in sap extract and ultrathin sections (**D** and **E)** from *M. sylvestris* leaves infected by *Malva vein clearing virus* DS-Ba-01 and Napoli isolates. Cylindrical inclusions, consisting in pinwheels (Pw), scrolls (Sc) and tubes (Tu), were observed in ultrathin sections for both MVCV isolates. Reference bars correspond to 200 nm.

All plant species belonging to the Malvaceae family, inoculated mechanically by both DS-Ba-01 and Napoli isolates, reacted showing initially vein clearing symptoms, followed by mosaic with different intensity and brightness, depending on the species. No symptoms were observed either locally or in the upper non-inoculated leaves in species from other botanical families. DS-Ba-01-specific riboprobe was used to check for virus latent infection in mechanically inoculated plants (Fig. 2).

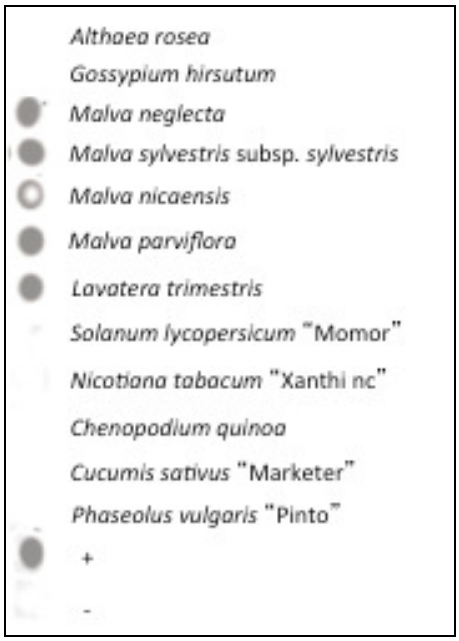

Fig. 2 - Molecular detection of MVCV in mecanically inoculated host plants by dot blot hybridization with the specific riboprobe produced in the present study. + = pMVCV plasmid; - = extract from healthy plant.

DS-Ba-01 and Napoli host ranges were identical and all malvaceous species become infected with the exception of *Gossypium hirsutum* and *Althaea rosea*. These results are in agreement with those reported for the MVCV isolate described for the first time in Germany (Hein, 1956) and in California (Costa and Duffus, 1957), suggesting that among malvaceous species, *Gossypium hirsutum* and *Althaea rosea* should be considered differential host plants for MVCV. However, symptoms observed in *Malva parviflora* in California consisted only in a very striking yellow mosaic limited to the main and secondary veins, with extent of symptoms only to the areas immediately adjacent to the veins (Costa and Duffus, 1957), whereas DS-Ba-01 and Napoli isolates, in the same plant species elicited symptoms consisting of larger yellow mosaic, covering most of the leaf surface (not shown). The evolution of symptomatology during the time induced by MVCV in malvaceous plant species was clearly documented by Hein (1956). The yellow mosaic areas became tendentially more evident in older plants, because of the widening and confluence of smallest mosaicated leaf areas (Hein, 1956). Thus, symptoms observed in the two cases probably could depend on the period of sampling, rather than to differences due to the variability among MVCV isolates. Moreover, particles length of DS-Ba-01 and Napoli isolates

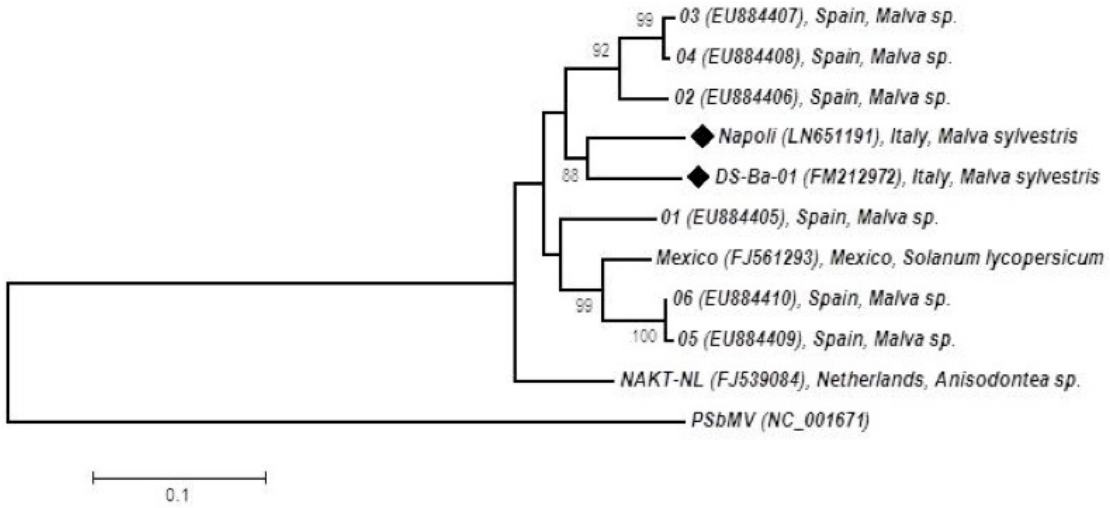

Fig. 3 - Maximum-likelihood tree showing phylogenetic relathionships among ten MVCV isolates based on nucleotide alignments of the coat protein (CP) gene. The positions of DS-Ba-01 and Napoli isolates are indicated by ♦ symbol. Bootsrap statistical analysis was carried out with 1000 replicates (only values ≥75% are shown). Tree was rooted by using *Pea seed borne mosaic virus* CP gene (Acc. N. NC001671) as outgroup.

were also in the same range of those described for MVCV isolates from Hungary, Croatia (formerly Yugoslavia) and Italy (Horváth *et al*., 1979; Martelli *et al*., 1969) and cytopathology described other Italian isolate sampled in the same region of DS-Ba-01, also indicated in this case type I cylindrical inclusions associated to MVCV infection (Martelli *et al*., 1969). Similar inclusions, consisting mostly in pinwheels and laminated aggregates, were described by Pisi et al., (1988) in *M. sylvestris* plants cultivated in North Italy (Emilia Romagna region) and found naturally infected by a potyvirus, most likely MVCV.

Cloning, sequencing and sequence analysis of the 3' 1.8 kb showed that the region contained the C-terminal part of the polymerase (NIb), the CP, and the 3´non-coding region (Acc. N. FM212972, isolate DS-Ba-01; Acc. N. LN651191, isolate Napoli). Percent nucleotide identity between DS-Ba-01 and Napoli isolates was, 89.7% and 90.9% for the 1.8 kb fragment and CP gene, respectively. The proposed CP N-terminal methionine was based on the possible occurrence of each amino acid to each position around the NIb (RNA-dependent RNA polymerase)/CP cleavage site for 113 *Potyviridae* species. The aminoacid sequence context was LFVYNQ/MDE for both DS-Ba-01 and Napoli, whereas 80% of the amino acid sequence consensus reported around the potyviral NIb–CP junction is V(I)-XH(L,F)-Q/A(S,G) (Adams *et al*., 2005). The putative motif in the CP sequence, associated with aphid transmissibility, was located at residues 6-8 downstream from the CP N-terminus and consisted of NAG (Asparagine-Alanine-Glicine) motif, as reported for another member of *Potyvirus* genus (i.e. *Bean yellow mosaic virus*) (Wylie *et al*., 2002). The motif SG-$(X)_3$-T-$(X)_3$-NT-$(X)_{30}$-GDD, conserved in the NIb gene of plant positive stranded viral RNA-dependent RNA polymerases (Domier *et al*., 1987; Kamer and Argos, 1984), was also present in the portion of DS-Ba-01 and Napoli NIb gene. The last six amino acids at 3'-end were GMRGVQ, located before the TGA opal codon. The 3' non-translated region consisted of 290 and 292 nucleotides for DS-Ba-01 and Napoli isolates, respectively.

Finally, phylogenetic relationships based on CP gene variability of 10 MVCV isolates from 4 different countries and mainly from malvaceous species (Fig. 3), did not show any significant grouping among MVCV isolates, indicating a low level of variability and selection among MVCV isolates characterized until now.

Common mallow is grown as an ornamental plant for its attractive flowers, but also for its medicinal properties widely recognized by modern phytotherapy (Heinrich *et al*., 2012). There are very few information about the epidemiology and incidence of MVCV in the common mallow cultivations and further studies are needed in order to clarify the quantitative and qualitative impacts of MVCV infections on common mallow crops. The use of high sensitive methods for MVCV detection, as the specific riboprobe developed in the present work, could be used to carry out the studies above mentioned since no specific antisera are commercially available against MVCV.

Competing interests

The authors declare that they have no competing interests.

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